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Evidence for Preferential Genotyping of a Minority Human Immunodeficiency Virus Population Due to Primer-Template Mismatching during PCR-Based Amplification

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Human immunodeficiency virus type 1 (HIV-1) genotyping assays have come to be widely used for monitoring antiretroviral drug resistance. We report a case in which primer-template mismatches during nested PCR-based amplification biased the composition of the original viral population in the sample, magnifying a distinct minority HIV-1 population. This observation might help to explain some unexpected HIV-1 genotypes.

Antiretroviral treatment of human immunodeficiency virus type 1 (HIV-1) infection does not successfully suppress HIV replication in all patients. Continued viral replication in the presence of selective drug pressure leads to antiretroviral resistance, hindering subsequent treatment efficacy. Drug resistance testing has been incorporated into the management of HIV-1-infected individuals in the last few years (9, 19). HIV-1 gene sequencing with various automated technologies is widely applied to detect drug resistance mutations. The proper use of genotypic resistance testing can improve the virological outcome among HIV-infected individuals (2, 4, 7, 23). Nevertheless, drug resistance testing and its clinical interpretation need to be refined.

One of the limitations is intrinsically related to the high genetic variability of HIV-1 (5, 20). Conventional population-based sequencing approaches allow characterization of the dominant viral species in the plasma sample. However, more sensitive techniques have demonstrated that complex and diverse mixtures of viral populations are frequently found in drug-experienced patients (16, 18), including distinct viruses as a results of dual infection (12, 13). In this context, perfect annealing of the PCR primers used in genotypic assays is not guaranteed, resulting either in lack of amplification of a certain viral variant or in a sequence that is not representative of the majority viral population in the plasma specimen. The latter might confound important therapeutic decisions that could be made on the basis of such genotypes.

Here we report a discordant genotypic characterization of the HIV-1 protease (PR) coding region on the basis of the sequence results obtained from a single reverse transcription (RT)-PCR and a subsequent nested PCR. Supernatant fluids from a primary culture of HIV-1-infected peripheral blood mononuclear cells were used to extract viral RNA. Incidentally, this method is not the usual practice in routine HIV drug resistance testing, which generally starts with patient plasma. The cells belonged to a 38-year-old homosexual man who first tested positive for HIV-1 in 1990. At the time the sample was taken, his plasma viremia was 4.5 $\log_{10}$ RNA copies/ml and his CD4 cell count was 80/mm^3. He had been heavily treated with several mono- and bitherapies and many different highly active antiretroviral therapies, including up to seven simultaneous drugs, at the time the sample was obtained. Viral RNA was reverse transcribed and PCR amplified in one step with primers 2146PR (5′-CAG AGC CAA CAG CCC CAC C-3′; positions 2147 to 2165 relative to HXB2CG) (14) and 3387RT (5′-AGT GCT TTG GTT CCT CTA AGG AGT TTA C-3′; positions 3388 to 3415).

A nested PCR of the PR coding region was subsequently performed with primers PR2212 (5′-AGC AGG AGC CGA TAG ACA-3′; positions 2213 to 2230) and PR2571 (5′-CCT GGC TTT AAT TTT ACT GG-3′; positions 2574 to 2593). Products obtained from either the first or the nested PCR amplification were sequenced by dRhodamine terminator cycle sequencing (Applied Biosystems) and electrophoresis in an automated sequencer (ABI 310; Applied Biosystems).

Both the first PCR product (containing the PR coding region and reverse transcriptase) and the product of the nested PCR (containing only the PR coding region) were independently cloned into a pGEM vector (Promega). Nine to 12 clones of each were sequenced by using the T7 and SP6 primers, for which hybridizing sites were contained in the cloning vector. We then compared the population-based sequence with the PR-cloned derived sequences from the first and nested PCR amplifications.

The population-based genotype of the PR coding region, directly obtained from the RT-PCR product, corresponded to a virus (termed here virus variant A) distinct from that obtained from the nested PCR product (variant B). According to the population-based sequences of the PR coding region, the

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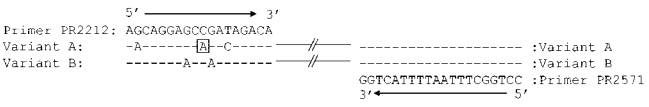

FIG. 1. Alignment of the sequences of the primers used in the nested PCR and virus variants A and B. Dashes indicate identity. All changes are G to T, slightly destabilizing the base pair mismatch, with the exception of the boxed nucleotide, which corresponds to a C-to-T change that significantly destabilizes the base pair mismatch.

drug resistance-associated mutations (11) were as follows: variant A, L10I, K20R, L33F, M46I, A71T, G73A, V77I, I84V, and L90M; variant B, L10I, V32I, L33F, G48V, I54V, A71V, and V82A. Of note, these genotypes did not show significant nucleotide mixture throughout the sequence, in clear correspondence to theoretical dominant viral populations. This discordance between genotypes from the same HIV-1 RNA sample prompted us to further analyze the population structure of both PCR products. Thus, 78% of the PR genotypes that resulted from the cloning of the RT-PCR product matched the sequence of variant A, another 11% resulted to be variant B, and the other 11% corresponded to recombinant forms. Similarly, 67% of the clones from the nested PCR that specifically amplified the PR coding region matched variant B, 8% matched variant A, and the rest corresponded to A-B recombinant species. The PR coding sequences of both virus variants belonged to subtype B. We calculated the genetic distances among 10 clonal sequences in the 3′ end of Gag, PR, and the beginning of the reverse transcriptase coding regions. The mean ($\pm$ the standard error) nucleotide distance between variants was 13.6% $\pm$ 1.4%, while the intravariant distances were $<$1% in both cases. These values strongly support the idea that the two variants are in fact different viruses that originally either coinfected or superinfected the patient's peripheral blood mononuclear cells. Although the goal of this study was never to look for dual infection, it shows that the minority viral population preferentially amplified in the nested PCR was genotypically unrelated to the viral population amplified in the first PCR.

Analysis of 10 clones consistently showed that the primer binding site in variant A had three mismatches with respect to the sequence of PR2212, while the primer binding site in variant B had only two (Fig. 1). The predicted priming efficiency of PR2212 binding to the target sequence of variant A would be 8.5% lower than for the target sequence of variant B. This value was derived from an algorithm that considers mismatches, duplex stability, bulge loops, and the distance of these elements from the primer's 3′ end (Oligo primer analysis software v6.65). Furthermore, from a qualitative point of view, the base pair mismatches between the primer and the template DNA for variant B were more stable than those between the primer and the template DNA for variant A (10). Reverse primer PR2571 had complete annealing with DNA templates from both variants A and B. None of the primers used overlapped cleavage sites in the Gag or Pol open reading frame. On the other hand, Gag substitutions previously suggested to coevolve with PR resistance mutations were unlikely to differentially influence primer binding efficiencies. Thus, codon 453 of Gag, whose second and third nucleotides overlap the first two nucleotides (5′ end) of primer 2146PR, did not show differences between variants. Clonal analysis showed that the binding site was also accessible for primer 2146PR in both viral variants without sequence variability that precludes efficient annealing. Therefore, primer PR2212 mismatching was the most plausible explanation for the preferential genotyping of a minority distinct HIV population during the nested PCR-based amplification.

Primer-template mismatches, especially if close to the 3′ end of the primer, may affect PCR product yield (1, 3, 15, 24). However, multiple internal mismatches might affect the specificity, rather than the efficacy, of the PCR, leading to a genotype that may not be representative of the dominant viral population. Although the primers used in drug resistance testing are intended to be as conserved as possible, natural HIV-1 diversity does continuously challenge oligonucleotide specificity. Besides other technical limitations of drug resistance assays that involve dideoxynucleotide sequencing (8, 21, 22), we have reported here a case in which primer-template mismatches on DNA amplification contributed to the chemical selection of a distinct minority HIV-1 population.

In conclusion, the genetic variability of HIV-1 may result in preferential annealing of the PCR-based amplification primers for unrelated virus variants present as minority populations in the sample, potentially as a result of superinfection or coinfection phenomena (12, 13). This observation might contribute to explaining unexpected HIV-1 population-based genotypes as those being wild type during viral breakthrough during antiviral therapy failure (6, 17).

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REFERENCES

1. **Ayyadevara, S., J. J. Thaden, and R. J. Shmookler Reis.** 2000. Discrimination of primer 3′-nucleotide mismatch by *Taq* DNA polymerase during polymerase chain reaction. Anal. Biochem. **284:**11–18.
2. **Baxter, J. D., D. L. Mayers, D. N. Wentworth, J. D. Neaton, M. L. Hoover, M. A. Winters, S. B. Mannheimer, M. A. Thompson, D. I. Abrams, B. J. Brizz, J. P. Ioannidis, and T. C. Merigan.** 2000. A randomized study of antiretroviral management based on plasma genotypic antiretroviral resistance testing in patients failing therapy. AIDS **14:**F83–F93.
3. **Christopherson, C., J. Sninsky, and S. Kwok.** 1997. The effects of internal primer-template mismatches on RT-PCR: HIV-1 model studies. Nucleic Acids Res. **25:**654–658.
4. **Cingolani, A., A. Antinori, M. G. Rizzo, R. Murri, A. Ammassari, F. Baldini, S. Di Giambenedetto, R. Cauda, and A. De Luca.** 2002. Usefulness of monitoring HIV drug resistance and adherence in individuals failing highly active antiretroviral therapy: a randomized study (ARGENTA). AIDS **16:**369–379.
5. **Coffin, J. M.** 1995. HIV population dynamics in vivo: implications for genetic variation, pathogenesis, and therapy. Science **267:**483–489.
6. **Descamps, D., P. Flandre, V. Calvez, G. Peytavin, V. Meiffredy, G. Collin, C. Delaugerre, S. Robert-Delmas, B. Bazin, J. P. Aboulker, G. Pialoux, F. Raffi, and F. Brun-Vézinet.** 2000. Mechanisms of virologic failure in previously untreated HIV-infected patients from a trial of induction-maintenance therapy. JAMA **283:**205–211.
7. **Durant, J., P. Clevenbergh, P. Halfon, P. Delgiudice, S. Porsin, P. Simonet, N. Montagne, C. A. Boucher, J. M. Schapiro, and P. Dellamonica.** 1999. Drug-resistance genotyping in HIV-1 therapy: the VIRADAPT randomised controlled trial. Lancet **353:**2195–2199.
8. **Galli, R. A., B. Sattha, B. Wynhoven, M. V. O'Shaughnessy, and P. R. Harrigan.** 2003. Sources and magnitude of intralaboratory variability in a sequence-based genotypic assay for human immunodeficiency virus type 1 drug resistance. J. Clin. Microbiol. **41:**2900–2907.
9. **Hirsch, M. S., F. Brun-Vezinet, B. Clotet, B. Conway, D. R. Kuritzkes, R. T. D'Aquila, L. M. Demeter, S. M. Hammer, V. A. Johnson, C. Loveday, J. W. Mellors, D. M. Jacobsen, and D. D. Richman.** 2003. Antiretroviral drug resistance testing in adults infected with human immunodeficiency virus type

1: 2003 recommendations of an International AIDS Society-USA panel. Clin. Infect. Dis. **37:**113–128.
10. **Ikuta, S., K. Takagi, R. B. Wallace, and K. Itakura.** 1987. Dissociation kinetics of 19 base paired oligonucleotide-DNA duplexes containing different single mismatched base pairs. Nucleic Acids Res. **15:**797–811.
11. **Johnson, V. A., F. Brun-Vezinet, B. Clotet, B. Conway, R. T. D'Aquila, L. M. Demeter, D. R. Kuritzkes, D. Pillay, J. M. Schapiro, A. Telenti, and D. D. Richman.** 2003. Drug resistance mutations in HIV-1. Top. HIV Med. **11:** 215–221.
12. **Jost, S., M. C. Bernard, L. Kaiser, S. Yerly, B. Hirschel, A. Samri, B. Autran, L. E. Goh, and L. Perrin.** 2002. A patient with HIV-1 superinfection. N. Engl. J. Med. **347:**731–736.
13. **Koelsch, K. K., D. M. Smith, S. J. Little, C. C. Ignacio, T. R. Macaranas, A. J. Brown, C. J. Petropoulos, D. D. Richman, and J. K. Wong.** 2003. Clade B HIV-1 superinfection with wild-type virus after primary infection with drug-resistant clade B virus. AIDS **17:**F11–F16.
14. **Korber, B. T., B. T. Foley, K. L. Kuiken, S. K. Pillai, and J. G. Sodroski.** 1998. Numbering positions in HIV relative to HXB2CG, p. 102–111. *In* C. Kuiken, B. Foley, B. Hahn, F. McCutchan, J. Mellors, and S. Wolinsky (ed.), HIV sequence compendium 1998. Theoretical Biology and Biophysics Group, Los Alamos, N.Mex.
15. **Kwok, S., D. E. Kellogg, N. McKinney, D. Spasic, L. Goda, C. Levenson, and J. J. Sninsky.** 1990. Effects of primer-template mismatches on the polymerase chain reaction: human immunodeficiency virus type 1 model studies. Nucleic Acids Res. **18:**999–1005.
16. **Martinez-Picado, J., M. P. DePasquale, N. Kartsonis, G. J. Hanna, J. Wong, D. Finzi, E. Rosenberg, H. F. Gunthard, L. Sutton, A. Savara, C. J. Petropoulos, N. Hellmann, B. D. Walker, D. D. Richman, R. Siliciano, and R. T. D'Aquila.** 2000. Antiretroviral resistance during successful therapy of HIV type 1 infection. Proc. Natl. Acad. Sci. USA **97:**10948–10953.
17. **Martinez-Picado, J., E. Negredo, L. Ruiz, A. Shintani, C. R. Fumaz, C. Zala, P. Domingo, J. Vilaró, J. M. Llibre, P. Viciana, K. Hertogs, C. Boucher, R. T. D'Aquila, and B. Clotet.** 2003. Alternation of antiretroviral drug regimens for HIV infection. A randomized, controlled trial. Ann. Intern. Med. **139:** 81–89.
18. **Martinez-Picado, J., L. Sutton, M. P. De Pasquale, A. V. Savara, and R. T. D'Aquila.** 1999. Human immunodeficiency virus type 1 cloning vectors for antiretroviral resistance testing. J. Clin. Microbiol. **37:**2943–2951.
19. **Richman, D. D.** 2004. Benefits and limitations of testing for resistance to HIV drugs. J. Antimicrob. Chemother. **53:**555–557.
20. **Saag, M. S., B. H. Hahn, J. Gibbons, Y. Li, E. S. Parks, W. P. Parks, and G. M. Shaw.** 1988. Extensive variation of human immunodeficiency virus type-1 in vivo. Nature **334:**440–444.
21. **Schuurman, R., L. Demeter, P. Reichelderfer, J. Tijnagel, T. de Groot, and C. Boucher.** 1999. Worldwide evaluation of DNA sequencing approaches for identification of drug resistance mutations in the human immunodeficiency virus type 1 reverse transcriptase. J. Clin. Microbiol. **37:**2291–2296.
22. **Shafer, R. W., K. Hertogs, A. R. Zolopa, A. Warford, S. Bloor, B. J. Betts, T. C. Merigan, R. Harrigan, and B. A. Larder.** 2001. High degree of interlaboratory reproducibility of human immunodeficiency virus type 1 protease and reverse transcriptase sequencing of plasma samples from heavily treated patients. J. Clin. Microbiol. **39:**1522–1529.
23. **Tural, C., L. Ruiz, C. Holtzer, J. Schapiro, P. Viciana, J. González, P. Domingo, C. Boucher, C. Rey-Joly, and B. Clotet.** 2002. Clinical utility of HIV-1 genotyping and expert advice: the Havana trial. AIDS **16:**209–218.
24. **Waterfall, C. M., R. Eisenthal, and B. D. Cobb.** 2002. Kinetic characterisation of primer mismatches in allele-specific PCR: a quantitative assessment. Biochem. Biophys. Res. Commun. **299:**715–722.